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DUSP6 is a novel transcriptional target of p53 and regulates p53-mediated apoptosis by modulating expression levels of Bcl-2 family proteins

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ABSTRACT

p53 regulates various cellular responses through transcriptional regulation of distinct sets of target genes. Dual specificity phosphatase 6 (DUSP6) is a cytosolic phosphatase that inactivates the extracellular-signal-regulated kinase 1/2 (ERK1/2). This study demonstrates that p53 transactivates DUSP6 in human colorectal HCT116 cells to regulate ERK1/2 in p53-mediated cell death. DUSP6 is transactivated by p53 overexpression and genotoxic agents, and chromatin immunoprecipitation revealed two p53-binding sites in the DUSP6 promoter responsible for DUSP6 induction. Expression of shDUSP6 inhibited 5′-FU-induced cell death, whereas overexpression of DUSP6 increased susceptibility to 5′-FU. 5′-FU treatment dephosphorylated ERK in a DUSP6-dependent manner, resulting in destabilization of Bcl-2 and stabilization of Bad. These results provide insights on the modulatory role of p53 in the survival pathway by up-regulating DUSP6.

1. Introduction

The p53 tumor suppressor plays a key role in the regulation of the cell cycle, DNA repair [1] and apoptosis [2]. Its activity is mainly mediated through its role as a transcription factor that activates or inhibits the transcription of the target genes [3]. Identification of the p53 target genes that are involved in cell death or the regulation of cell survival is necessary to define the molecular mechanism(s) by which p53 functions as a tumor suppressor under various cellular stresses.

Dual specificity phosphatase 6 (DUSP6) is one member of the family of mitogen-activated protein kinase (MAPK) phosphatases (MKPs) or cysteine-dependent DUSPs comprised of 10 catalytically active proteins that share a common structure at their C-terminal catalytic domain. DUSP6 specifically dephosphorylates extracellular-signal-regulated kinase (ERK), which distinguishes it from other MAPK phosphatases such as DUSP1 and DUSP4, which instead exhibit broad substrate specificities for c-Jun N-terminal kinase (JNK), p38, and ERK [4,5].

DUSP6 is a negative regulator of ERK stimulated with fibroblast growth factor (FGF) during mouse development [6]. Since ERK is a key effector MAPK involved in the RAS-GTP signaling pathway in response to signals from growth factor-receptor tyrosine kinase, the regulation of ERK by DUSP6 could be crucial for deactivating the survival signals stimulated by growth factors and determining the cell fate in physiological conditions [7,8]. Therefore, inactivation of DUSP6 may abrogate the negative controlling pathway of ERK and, subsequently, uncontrolled cell growth, which in turn could lead to tumorigenesis and progression. Such cases have been reported in pancreatic cancer cells, which show the gain-of-function mutations in the K-RAS2 gene [9,10]. DUSP6 expression was significantly decreased in the invasive carcinoma cells with pancreatic invasive carcinomas [11], in primary lung cancers [12], and in ovarian cancers [13]. Furthermore, restoration of DUSP6 expression suppresses cellular proliferation in lung cancer cells [12] and in ovarian cancer cells [13], impairs epithelial to mesenchymal transition and cell invasion in esophageal squamous cell carcinoma cells [14], and sensitizes ovarian cancer cells to cisplatin-induced apoptosis [13]. However, these tumor suppressive roles of DUSP6 are complicated by observation that DUSP6 expression levels were increased in other tumors like melanoma [15], breast cancers [16], and glioma [17]. These contradictory roles of DUSP6 in tumorigenesis are not clearly understood yet.

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Besides roles of DUSP6 in tumorigenesis, several reports showed that DUSP6 influences cell death by regulating ERK activity. For instance, ERK1/2 phosphorylates Bad, a pro-apoptotic Blc-2 family protein that disrupts the interaction with Bcl-$_{xL}$, protecting cells from apoptosis. However, DUSP6 inactivates ERK1/2 such that unphosphorylated Bad can associate with Bcl-$_{xL}$, leading to the cell death [18]. Also, ERK1/2 can phosphorylate Bcl-2, and the phosphorylated Bcl-2 protein is resistant to proteosomal degradation, protecting cells from apoptosis [19]. This protection can be inhibited by DUSP6 expression through the inactivation of ERK1/2.

Here, we explored the potential p53 target genes that are associated with cell death, and found that DUSP6 is a novel target gene of p53, promoting the cell death in response to genotoxic stresses by modulating ERK phosphorylation and the expression levels of Bcl-2 family proteins such as Bad, Bcl-2 and Bcl-$_{xL}$.

2. Materials and methods

2.1. Cell culture and reagents

HCT116 human colorectal carcinoma parental cells and p53-deficient cells were maintained in McCoy's 5A medium with 10% fetal bovine serum (FBS) supplemented with 2 mM L-glutamate, 100 U/mL Penicillin and 100 μg/mL Streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was generated as described previously [20]. 5-Flurouracil (5-FU) and Etoposide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Blasticidin was purchased from Invitrogen Life Technologies (Valencia, CA, USA). Anti-actin antibody was purchased from Chemicon International (Temacula, CA, USA). Anti-DUSP6 antibody was purchased from Abnova (Taipei, Taiwan). Anti-p53 antibody was purchased from Oncogene Science (Cambridge, MA, USA). Anti-p21 antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-pERK1/2 and total anti-ERK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit/-mouse IgGs were purchased from Jackson ImmunoResearch Lab (West Grove, PA, USA).

2.2. Infection of adenovirus

HCT116 p53$^{-/-}$ cells were infected with the recombinant adenoviral vector expressing wild-type p53 (Ad-p53) or wild type adenovirus (Ad-WT; as the negative control). The multiplicity of infection (MOI) was defined as the ratio of infectious virus particles to cells. The titer of the virus was calculated using the standard issue Culture Infectious Dose 50 ($TCID_{50}$) following the manufacturer's protocol (Ad Easy™ vector system; Qbiogene, Montreal, PQ, Canada).

2.3. Construction of short hairpin RNAs (shRNAs) and analysis of apoptotic cell death

Plasmid vector pcDNA6A:GFP::H1 (indicated as vector in the text and figures) was designed to express green fluorescent protein (GFP) and shRNA under the cytomegalovirus promoter and H1 promoter, respectively. The pcDNA6A:GFP::H1:shDUSP6 expressing shDUSP6 (indicated as shDUSP6 in the text and figures) was generated by annealing the shDUSP6 oligonucleotides (generating overhang *Bgl*II and *Cla*I sites) and then ligating the annealed shDUSP6 oligonucleotides into *Bgl*II and *Cla*I sites of pcDNA6A:GFP:H1. The oligonucleotides used for generation of shDUSP6 are listed in Table 1. HCT116 parental cells and HCT116 p53$^{-/-}$ cells were transfected with either pcDNA6A:GFP::H1 vector or pcDNA6A:GFP::H1:shDUSP6 plasmids using Effectene® transfection reagent (Qiagen, Chatsworth, CA, USA) and transfected cells were selected with blasticidin (10 μg/mL). The cells were subjected to treatment with 5′-FU (200 μg/mL) or Etoposide (100 μg/mL) for the indicated times, and the cell death rates were monitored by XTT assay. The data represented as mean ± standard deviation (S.D.; $n = 3$). The knock-down efficiency was checked by western blotting.

Table 1
List of primers.

Primer names	Sequences
DUSP6-RT	CGAGTCTGACCTTGACCGA
	AGGCATCGTTCATCGACAG
GAPDH-RT	CCATCACCATCTTCCAGGAGCGAG
	TGCCAGTGAGCTTCCCGTTCAGCTC
p21-RT	CGACTGTGATGCGCTAAT
	CTTGGAGAAGATCAGCCG
p53-RT	GTGCCTTCCCAGAAAACCTA
	TCCGTGCCAGTAGATTACCA
shDUSP6	GATCCCCAAGACGGTGGCGTGGCTCAACTTCAAGAGAGTTG
	AGCCACGCCACCGTCTTTTTTTGGAAAT
	CGATTTCCAAAAAAAGACGGTGGCGTGGCTCAACTCTCTTGA
	AGTTGAGCCACGCCACCGTCTTGGG
DUSP6 ChIP 1	GCGCTGGACAAAGGGAGGA
(−1625 to −1425)	TGAGCGAGGTGTGCTGGGA
DUSP6 ChIP 2	AGCCTACATTTCCCAAACCAGGGA
(−2404 to −2125)	TAATCCGGCCTTCCCTCCCTAAA

2.4. DUSP6 over-expression

The full-length cDNA fragments of human DUSP6 amplified by PCR were cloned into N-terminal flag tagged PCR®8/GW/Topo® entry vector, and confirmed by DNA sequencing analysis. The open reading frame region of DUSP6 in the entry vector was subcloned into the N-terminal GFP-tagged destination vector following the Gateway® Technology protocol (Invitrogen). The construction was verified by automatic DNA sequencing. HCT116 parental cells and HCT116 p53$^{-/-}$ cells were transfected with either GFP:XB vector or GFP:DUSP6 using Effectene® (Qiagen) and transfected cells were selected with blasticidin (10 μg/mL). Transiently selected cells were subjected to treatment with 5′-FU (200 μg/mL) or Etoposide (100 μg/mL) for the indicated times.

2.5. Chromatin immunoprecipitation (ChIP) analysis

HCT116 parental (2×10^8) cells were seeded on 100 mm-diameter dish (Nunc) and were treated with 5′-FU (200 μg/mL) for indicated the times on the following day. For cross-linking the bound proteins to chromatin, the medium containing 1% formaldehyde (Sigma–Aldrich) was added drop-wise directly into the dish, which was then rotated gently at room temperature for 10 min. The cross-linked cells were resuspended in swelling buffer (25 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM dithiothreitol and freshly-added aforementioned protease inhibitor cocktail). Following 10 min incubation on ice, nuclei were released by homogenizing 20 times up-and-down. Nuclei were resuspended in ChIP lysis buffer (50 mM HEPES–KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate and the aforementioned protease inhibitors) and sonicated to shear the chromatins of average fragment size 500–1000 bp. The sheared protein–DNA complexes were immunoprecipitated with anti-p53 antibody (Oncogene Science) and captured on protein A Sepharose beads (GE Healthcare, Buckinghamshire, UK). After immunoprecipitation, the beads were washed three times with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–Cl pH 8.0) and once with final wash buffer (0.1% SDS, 1% Triton

X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–Cl pH 8.0). Finally, the chromatin complexes were eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$) and DNA–protein cross-linking was reversed by addition of proteinase K and heating at 65 °C overnight. The DNA was purified by phenol extraction followed by ethanol precipitation. PCR amplification of the DUSP6 gene was performed using the specific primers detailed in Table 1.

2.6. Statistical analysis

Values are presented as mean ± standard deviation. The paired Student's *t*-test was applied to determine whether the differences between values were significant ($P < 0.05$).

3. Results

3.1. p53 transactivates DUSP6 under genotoxic stresses

To identify novel targets that are transcriptionally activated by p53, we performed DNA chip analysis with p53-expressing adenovirus-infected HCT116 cells using commercial oligonucleotide DNA chips (Digital Genomics, Seoul, Korea). Several candidates including DUSP6 of p53 target genes were identified (data not shown). DUSP6 regulates proteins involved in both cell proliferation and cell death [6,8]. Thus, we focused on whether DUSP6 is involved in p53-mediated apoptosis. First, to verify the transactivation of DUSP6 by p53, HCT116 p53 parental cells were infected with the adenovirus expressing p53 (Ad p53) or the control adenovirus (Ad control). RT-PCR analysis revealed the substantial increase of DUSP6 mRNA in HCT116 p53 parental cells infected with Ad p53 compared to that in HCT116 p53 parental cells infected with Ad control (Fig. 1A). Furthermore, treatment of HCT116 WT cells with 5′-FU increased the endogenous p53 that results in the increased expression of DUSP6 mRNA; however, HCT116 $p53^{-/-}$ cells treated with 5′-FU showed no increase of DUSP6 mRNA (Fig. 1B). To further determine whether p53 was responsible for increasing the protein level of DUSP6 in response to different death stimuli, HCT116 parental cells and HCT116 $p53^{-/-}$ cells were treated with 5′-FU and Etoposide. Indeed, treatment of 5′-FU and Etoposide to HCT116 parental cells, but not to HCT116 $p53^{-/-}$ cells increased the DUSP6 protein level while p53 protein level and p21 protein (a best known p53 target gene) level were increased (Fig. 1C and D). These results implicate that DUSP6 is a novel transcriptional target of p53.

To probe for the potential p53 binding sites in the promoter region of DUSP6, computer analysis of the promoter region (about 2.5 kb from ATG translation start site) using MatInspector in Genomatix (http://www.genomatix.de/) was performed. This analysis revealed two putative binding sites at −1552/−1585 and −2316/−2349 within 2.5 kb of the DUSP6 promoter region (Fig. 2A). These putative binding sites contain the core region of the p53 binding consensus motif. To investigate whether or not p53 could occupy these putative binding sites in response to 5′-FU, the chromatin immunoprecipitation (ChIP) assay was performed. HCT116 parental cells were treated with 5′-FU, and ChIP and PCR were performed using anti-p53 antibody and primers comprising of −2404 to −2125 and −1625 to −1425, respectively (Fig. 2A). At 6 or 12 h after 5′-FU treatment, the putative p53 binding sites were occupied by p53 (Fig. 2B), suggesting that these putative p53 binding sites may be functional for DUSP6 expression in response to genotoxic stresses like 5′-FU.

3.2. DUSP6 plays a role in p53-mediated cell death induced by genotoxic stresses

The transactivation of DUSP6 by p53 under genotoxic stress suggests that DUSP6 may play a role in p53-mediated cell death. To determine the role of DUSP6 in p53-mediated cell death, we investigated the effects of the forced expression of DUSP6 on the cell viability in response to 5′-FU either in HCT116 parental cells or in HCT116 $p53^{-/-}$ cells. Ectopic expression of DUSP6 in HCT116 parental cells or in HCT116 $p53^{-/-}$ cells substantially

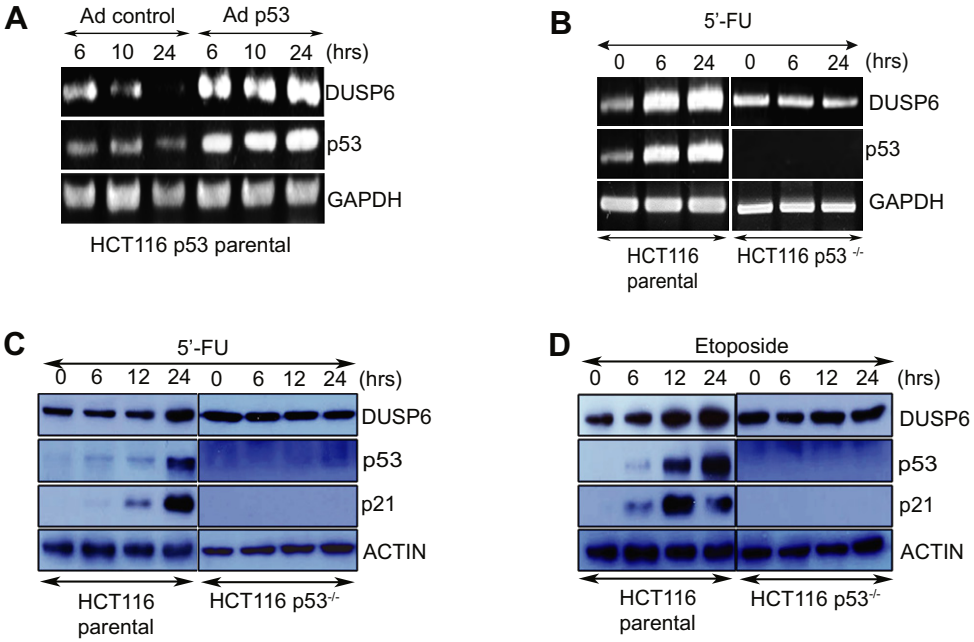

Fig. 1. p53 transactivates DUSP6 under genotoxic stresses. (A and B) HCT116 parental cells (A) were infected with wild type adenovirus (Ad control) or adenovirus expressing p53 (Ad p53) for 6, 10 and 24 h. HCT116 parental cells and HCT116 $p53^{-/-}$ cells (B) were treated with 5′-FU (200 μg/mL) for 0, 6 and 24 h. Then, mRNA levels of DUSP6, p53, and GAPDH were analyzed by RT-PCR. (C and D) HCT116 parental cells and HCT116 $p53^{-/-}$ cells were treated with 5′-FU (200 μg/mL) (C) or Etoposide (100 μg/mL) (D) for the indicated times, and whole cell lysates were prepared using RIPA buffer. The whole cell lysates were subjected to SDS–PAGE and probed with the indicated antibodies. The representative data were presented from three individual experiments.

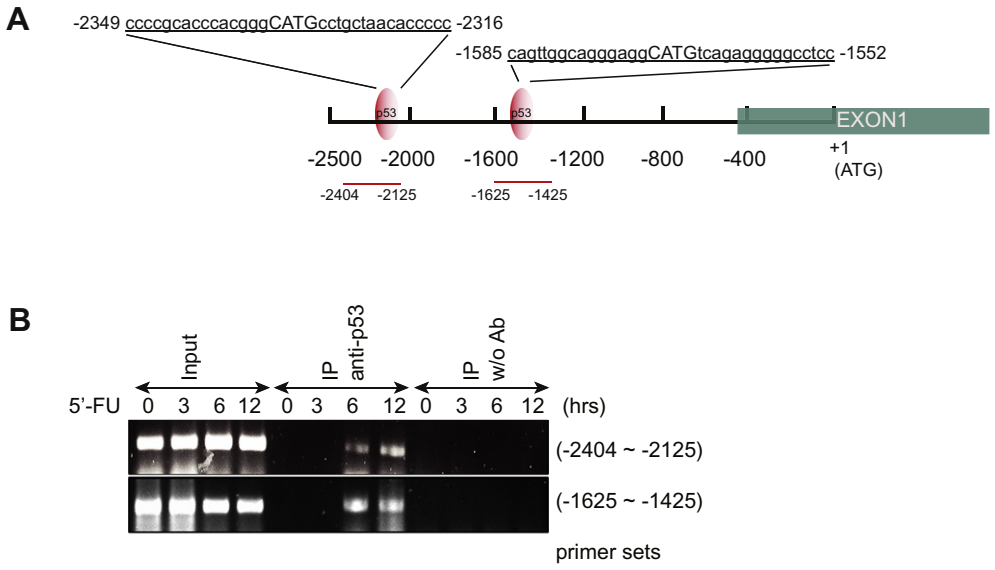

Fig. 2. DUSP6 promoter contains two putative p53 binding sites. (A) The schematic representation of two putative p53 binding sites in DUSP6 promoter region. (B) HCT116 cells were treated as indicated and were subjected to ChIP analysis. A p53-specific antibody was used to precipitate p53 bound to chromatin. The p53-associated DUSP6 promoter was PCR-amplified using the indicated primers. The representative data were presented from three individual experiments.

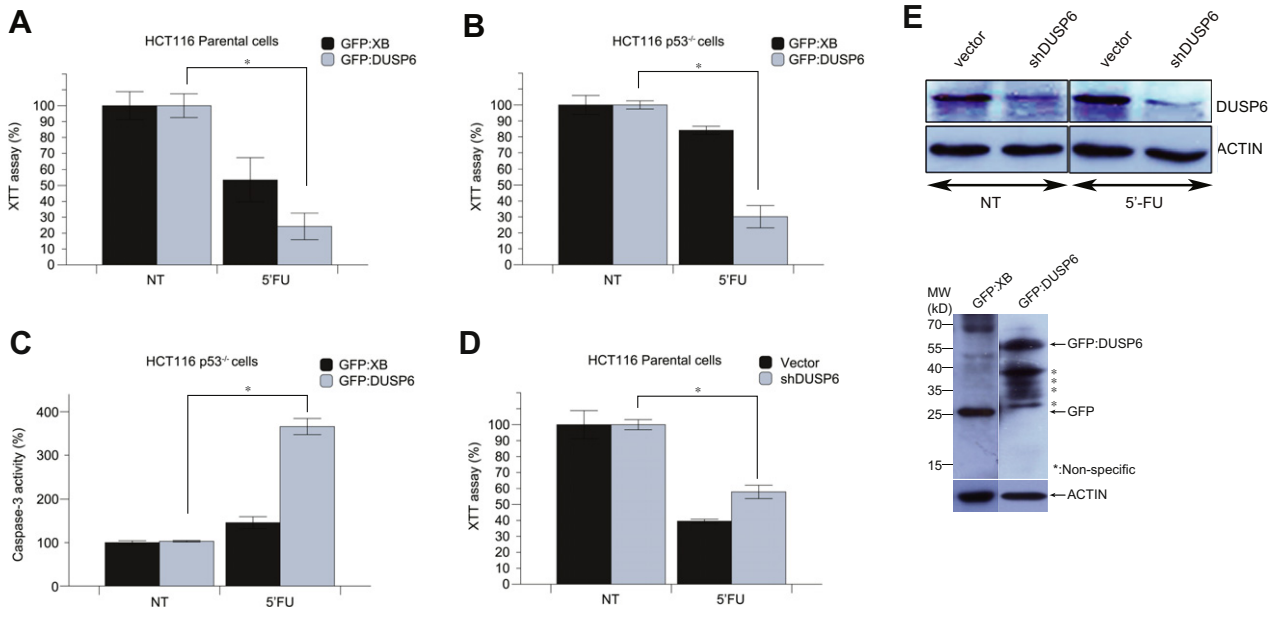

Fig. 3. DUSP6 is a necessary factor for p53-mediated cell death. (A–C) HCT116 parental cells (A) and HCT116 p53$^{-/-}$ cells (B) were transiently transfected with either control vector or GFP fused DUSP6 expression construct (indicated as DUSP6). Twenty-four hours after transfection, cells were treated with 5′-FU (200 μg/mL) for another 24 h. The viability was measured by XTT assay (A and B) or caspase-3 activity was measured using colorimetric DEVD-pNA substrate (C). (D and E) HCT116 parental cells were transfected with either vector control or shDUSP6 expression vector. The cells were treated with 5′-FU (200 μg/mL) for 24 h. The viability was measured by XTT assay (D). Immunoblottings using anti-DUSP6 and anti-actin antibodies (upper panel) or anti-GFP antibody (bottom panel) were carried out (E). Data were represented as mean ± standard deviation (S.D.; $n = 3$) (Student's t-test: $^{*}P < 0.001$.)

reduced the cell viability examined by XTT assays in response to 5′-FU (Fig. 3A and B). The reduction of cell viability by overexpression of DUSP6 in HCT116 p53$^{-/-}$ cells in response to 5′-FU appeared to be mainly due to apoptotic cell death because caspase-3 activity was greatly enhanced by DUSP6 expression (Fig. 3C). Ectopic expression of DUSP6 in HCT116 cells was confirmed by Western blot analysis (Fig. 3E)

To further confirm the role of DUSP6 in p53-mediated cell death, we examined the cell viability in HCT116 parental cells expressing DUSP6 shRNA (shDUSP6). The expression of DUSP6 observed by Western blot analysis was significantly reduced by shDUSP6 in both 5′-FU treated and untreated cells, but not by control vector (Fig. 3E). HCT116 parental cells expressing shDUSP6 displayed the increased cell viability induced by 5′-FU (Fig. 3D), Etoposide (Supplementary Fig. 1A) or TRAIL (Supplementary Fig. 1B); however, HCT116 p53$^{-/-}$ cells expressing shDUSP6 showed limited reduction of cell death in response to 5′-FU, since 5′-FU induces only limited, if any, death of HCT116 p53$^{-/-}$ cells

(data not shown). These results indicate that the induction of DUSP6 by p53 plays a critical role in genotoxic stresses and death ligand-induced cell death.

3.3. Induction of DUSP6 by p53 in response to 5′-FU is correlated with dephosphorylation of ERK and the levels of the Bcl-2 family proteins

The magnitude and duration of ERK activity determine the cellular proliferation and growth of tumor cells [21,22]. Since DUSP6 is a phosphatase specific for ERK dephosphorylation, we assumed that the transactivation of DUSP6 by p53 might regulate the ERK function in p53-mediated cell death. To show the functional link between ERK dephosphorylation and DUSP6 induction by p53 in p53-mediated cell death, DUSP6 induction and phosphorylation status of ERK were observed in HCT116 parental cells and HCT116 p53$^{-/-}$ cells treated with 5′-FU. As expected, in HCT116 parental cells, the ERK dephosphorylation was highly correlated with p53 induction and subsequent induction of DUSP6 (Fig. 4A). However, HCT116 p53$^{-/-}$ cells showed only marginal increase of ERK phosphorylation within 1–2 h after 5′-FU treatment. Conversely, ERK was significantly phosphorylated in HCT116 parental cells expressing shDUSP6 in response to 5′-FU (Fig. 4B). Since Bcl-2 has known to be phosphorylated by ERK [23], we examined the phosphorylation status of Bcl-2 in HCT116 parental cells transiently transfected with DUSP6 expression constructs. Control vector did affect a little or no changes of Bcl-2 phosphorylation status at Thr 56 and Ser 70, whereas DUSP6 expression provoked significant decrease of Ser 70 phosphorylation but not Thr 56 phosphorylation of Bcl-2 (Fig. 4C). Together, these results indicate that p53 transactivates DUSP6 that subsequently dephosphorylates ERK when cells are challenged with genotoxic stresses.

Activated ERK phosphorylates Bad and Bcl-2 at serine residues such that its pro-apoptotic function of Bad is masked, and Bcl-2 is protected from proteosomal degradation, leading to cell survival [18,23]. Thus, we assumed that DUSP6-mediated dephosphorylation of ERK may influence the expression profile of Bcl-2 family proteins. In this context, to investigate the changes of expression profiles of Bcl-2 family proteins, DUSP6 expression was suppressed by introducing shDUSP into HCT116 parental cells or HCT116 p53$^{-/-}$ cells. In both cells expressing shDUSP6, the expression levels of Bcl-2 and Bcl-$_{xL}$ were increased, whereas that of Bad was decreased (Fig. 5A). On the contrary, when DUSP6 was overexpressed in HCT116 parental cells or HCT116 p53$^{-/-}$ cells, the expression levels of Bcl-2 and Bcl-$_{xL}$ showed little decrease or no change in both cells, whereas that of Bad was significantly increased (Fig. 5B). These results confirm that DUSP6 determines the expression levels of Bcl-2 family proteins like Bcl-2, Bcl-$_{xL}$, and Bad by regulating ERK activity, and provides a functional significance of DUSP6 induction by p53 in p53-mediated apoptosis.

4. Discussion

Genotoxic stresses provoke cellular responses including cell cycle arrest coupled with DNA repair or cell death. The huge line

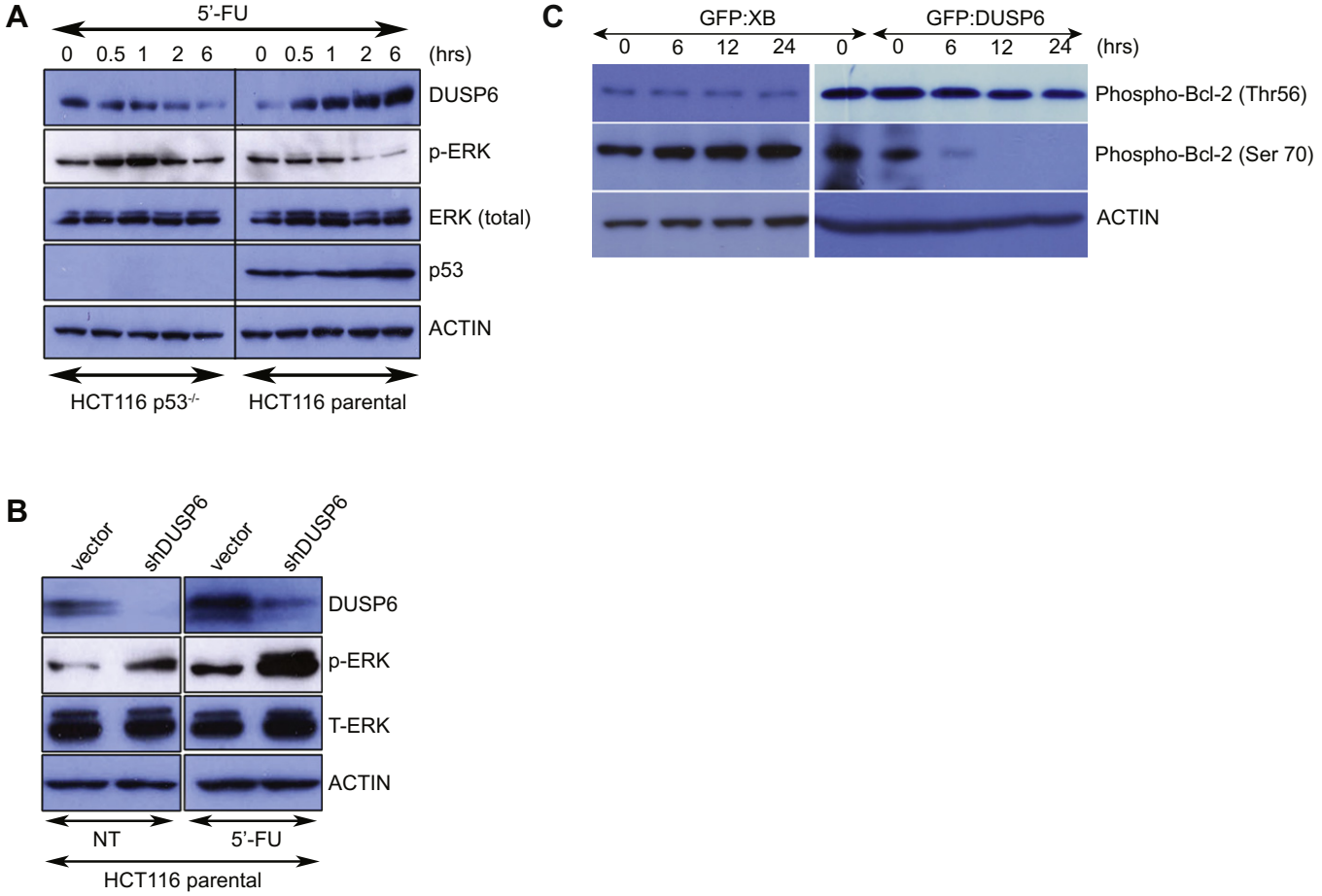

Fig. 4. DUSP6 dephosphorylates ERK in a p53-dependent manner. (A) HCT116 parental cells and HCT116 p53$^{-/-}$ cells were treated with 5′-FU (200 μg/mL) for the indicated time. Western blotting used anti-p53, anti-DUSP6 and anti-phospho-ERK (*p*-ERK) antibodies. (B) HCT116 parental cells were transfected with either pcDNA6A:GFP::H1 vector or pcDNA6A:GFP::H1:shDUSP6, and then the transfected cells were selected with blasticidin for 24 h. The cells were treated with 5′-FU (200 μg/mL) for 3 h. DUSP6, p-ERK and total ERK expression were analyzed by Western blotting. (C) HCT116 parental cells were transfected with either pGFP:XB or pGFP:DUSP6, and lysates were harvested at indicated times. Western blot analysis was performed using phopho-Bcl-2 antibodies. The representative data were presented from three individual experiments.

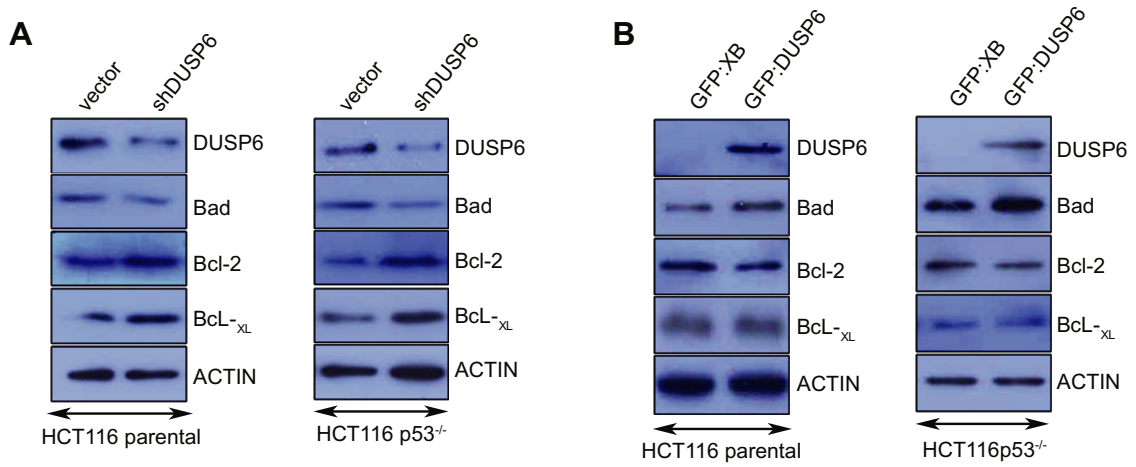

Fig. 5. DUSP6-dependent ERK dephosphorylation regulates the expression levels of Bad, Bcl-2, and BcL-$_{xL}$. (A) HCT116 parental cells and HCT116 p53$^{-/-}$ cells were transfected with either pcDNA6A:GFP::H1 vector or pcDNA6A:GFP::H1:shDUSP6. The whole cell lysates were probed with the indicated antibodies. (B) HCT116 parental cells and HCT116 p53$^{-/-}$ cells were transfected with either control vector or GFP:DUSP6. The whole cell lysates were probed with the indicated antibodies. The representative data were presented from three individual experiments.

of evidence supports that p53 plays a key role in determining the cell fate by transactivating distinct sets of target genes, leading to cell cycle arrest or cell death [3,24]. Presently, we show that p53 induces DUSP6, a key phosphotase for ERK, which enhances cell death by modulating ERK phosphorylation and the expression levels of Bcl-2 family proteins in response to genotoxic stresses.

Pro-death key target genes of p53 including Noxa [25,26], PUMA [27], PIG3 [28], Bax [29], ASC [30], Fas [31] and DR5 [32] have been identified and verified their roles in p53-mediated cell death. Other p53 target genes related to the growth inhibitory signaling pathways including DUSP1, DUSP2, DUSP4, and DUSP5, which can inactivate MAPKs like JNK, p38 or ERK, have been shown to play a

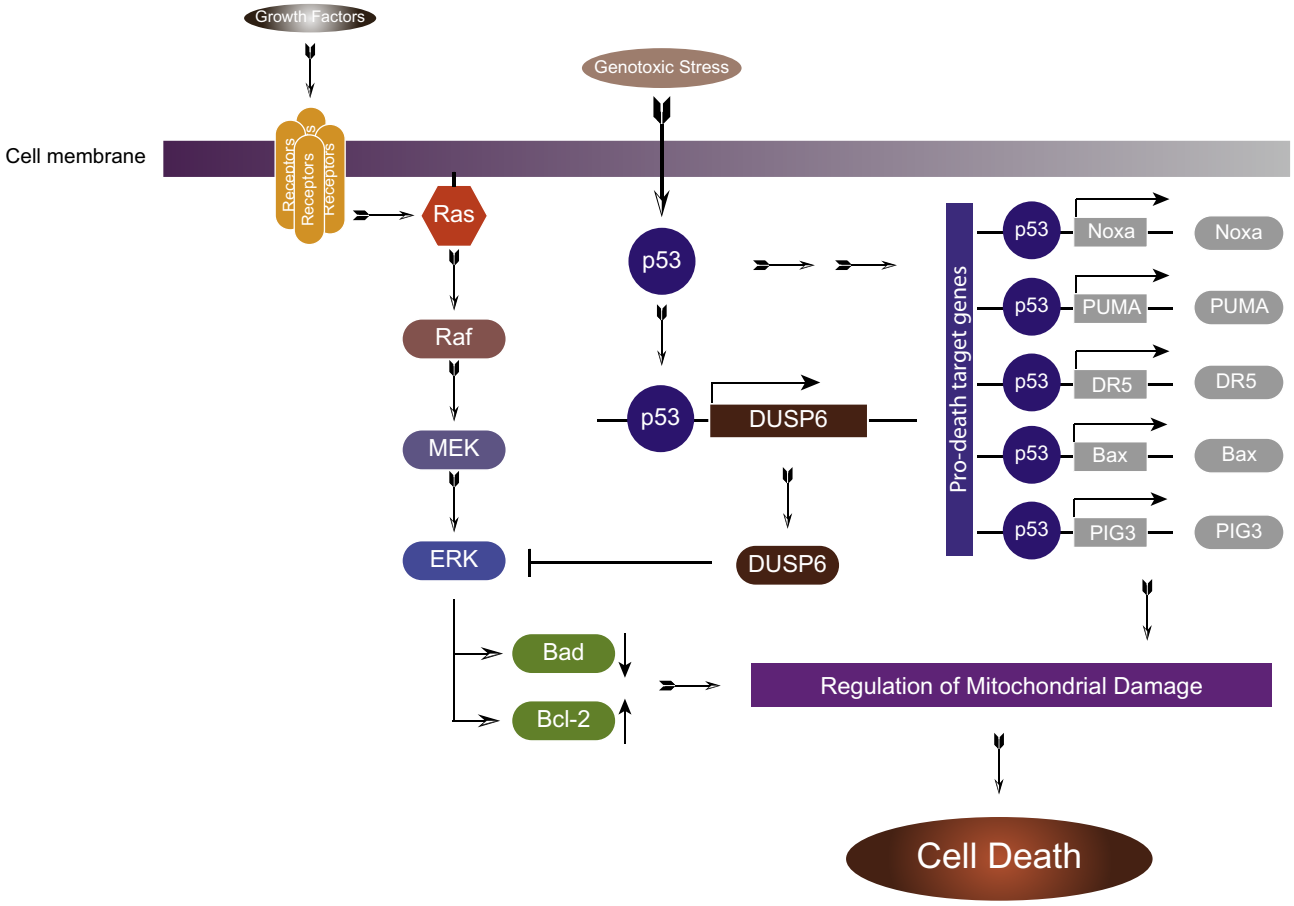

Fig. 6. Schematic diagram of the relationship between Ras/Raf/MEK/ERK survival axis and p53-mediated DUSP6 expression. p53 transactivates a number of target genes (e.g., Noxa, PUMA, DR5, Bax, and PIG3) that converge to enhance the cell death pathway. In addition, p53 can inhibit the cell survival pathway by transactivating its target genes like DUSP6 that can dephosphorylate and inactivate ERK1/2. Dephosphorylation of ERK1/2 results in degradation of Bcl-2 and in activation of Bad, leading to mitochondrial damage and ultimately cell death.

crucial role in p53-mediated cell death [33–37]. Also, it has reported that DUSP1 and DUSP2 can be transactivated by glucose deprivation or p53 during oxidative stress, leading to cell death [34,38]. The present study adds DUSP6 to the list of p53-induced target genes involved in inhibition of cell proliferation (Fig. 6).

Recent studies showed that E twenty six (Ets) 2 protein, the well-known target of ERK signaling, activates DUSP6 transcription by binding to the conserved binding site for Ets2 present in the upstream region of DUSP6 promoter in response to FGF [39,40] or in response to PMA [41] or in the intron 1 of DUSP6 gene in response to active ERK activity [42]. These results suggest a feedback loop between DUSP6 and ERK and Ets2 in a way that DUSP6 transcription is tightly regulated by Ets2 activity that is controlled by ERK activity [42]. Ets1 also binds to DUSP6 promoter region to promote DUSP6 transcription in lung cancer cells, and this elevation of DUSP6 expression is abrogated by inactivation of ERK activity [43]. This feedback regulatory loop can be also established between p53 and DUSP6 and Ets2. Our results indicate that p53 can induce DUSP6 expression. As mentioned above, Ets2 activity is regulated by ERK that is inhibited by DUSP6. Ets2 can also transactivate p53 by binding to Ets-binding sites in p53 promoter region, and can provoke apoptosis in the thymus of Ets2 transgenic mice or in Ets2-overexpressing HeLa cells. Ets2-induced apoptosis in the thymus of Ets2-transgenic mice can be rescued by crossing with p53-deficient mice, revealing that p53 is a key mediator for Ets2-induced apoptosis [44,45]. Thus, we suggest that Ets2 may be an activator for both p53 and DUSP6, whereas p53 and DUSP6 may be an inhibitor for Ets2 through ERK inactivation. The signaling axis of p53, DUSP6, ERK, and Ets2 may establish a feedback regulatory loop to determine the cell fate in response to DNA damage.

ERK has shown as a key player to protect cells from apoptosis by phosphorylating Bad and Bcl-2, resulting in the dissociation of Bad from Bcl-$_{xL}$ and protection of Bcl-2 from proteosomal degradation [18,19,46]. Our results showed that p53-dependent DUSP6 upregulation caused the dephosphorylation of ERK, affecting the expression levels of Bad, Bcl-2 and Bcl-$_{xL}$. Indeed, the phosphorylation status of Bcl-2 (Ser70) and the expression level of Bcl-2 protein are significantly decreased by DUSP6 expression (Figs. 4 and 5). These results emphasize the role of p53 in modulating the expression levels of Bcl-2 family proteins by regulating DUSP6 expression and its downstream substrate ERK. It is, also, of interesting to note that DUSP6 is upregulated by tumor necrosis factor-alpha (TNF-α) treatment, and cell death induced by TNF-α in HUVEC cells can be completely blocked by a DUSP6 dominant negative mutant form [23], indicating that modulation of DUSP6 expression could be a key step to regulate cell death in some cells induced by not only genotoxic stresses but also death ligands.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.10.031.

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